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Analytical Methods

Fast UPLC/PDA determination of squalene in Sicilian P.D.O. pistachio from Bronte: Optimization of oil extraction method and analytical characterization

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ABSTRACT

A fast reversed-phase UPLC method was developed for squalene determination in Sicilian pistachio samples that entry in the European register of the products with P.D.O. In the present study the SPE procedure was optimized for the squalene extraction prior to the UPLC/PDA analysis. The precision of the full analytical procedure was satisfactory and the mean recoveries were 92.8 ± 0.3% and 96.6 ± 0.1% for 25 and 50 mg L^{-1} level of addition, respectively. Selected chromatographic conditions allowed a very fast squalene determination; in fact it was well separated in ~0.54 min with good resolution. Squalene was detected in all the pistachio samples analyzed and the levels ranged from 55.45–226.34 mg kg^{-1}. Comparing our results with those of other studies it emerges that squalene contents in P.D.O. Sicilian pistachio samples, generally, were higher than those measured for other samples of different geographic origins.

1. Introduction

Pistachio (*Pistacia vera L.)* is a plant of the Anacardiaceae family, which probably originated in Central and Western Asia and nowadays is distributed throughout the Mediterranean basin (Gentile et al., 2007). The pistachio tree is mainly cultivated in Iran, Turkey, USA, Greece, Syria, Italy and Spain. In Italy, only a single pistachio variety (*Bianca*) is grown (Bellomo & Fallico, 2007; Fabbri & Valenti, 1998; Vitale et al., 2013) and its cultivation is concentrated mainly around Mount Etna, in the area of Bronte (Sicily), where the lava-rich soil and climate allow the production of a nut with intense green colour and aromatic taste that is very appreciated for its high quality in international markets. Given its singular traits, its nutritional and organoleptic properties, as well as its gorgeous taste and smell, on 9 June 2009 the European Union (EU) publishes the specification which gives to the "Green pistachio of Bronte" the Protected Designation of Origin (P.D.O.) (Council Regulation (EC) No. 510/2006). According this EU Regulation, the P.D.O. "Green pistachio from Bronte" is reserved for the product, in shell, shelled or peeled, of plants of the botanical species *Pistacia vera*, cultivar *Napoletana*, also called *Bianca* or *Nostrale*, grafted on *Pistacia terebinthus*. EU tutelage, as recognition of the special procedure for cultivation and biodiversity conservation, establishes the rules and regulations regarding the exact area of cultivation (i.e. the Sicilian municipalities of Bronte, Adrano and Biancavilla), and production, the harvesting and labelling practices; thus protecting not only product but also consumer. Annual production of pistachio from Bronte with its 30 tons, which represents 80% of the total Sicilian production (Gentile et al., 2007), is very poor in comparison to Asian and American ones; however, it is compensated by the very high quality of the product (Vitale et al., 2013).

Pistachio seeds consist of the shell and the edible kernel, which has a papery seed coat (skin). The chemical composition of the pistachio seeds is complex and obviously not completely known. The popularity of pistachio is mainly due to their nutritional components and the literature contains informations on the health effects of pistachio. In fact, it is reported that pistachio nuts are a rich source of phenolic compounds, known for their high antioxidant activity, and not only contained in the seeds, but also in the skin (Halvorsen et al., 2006). The popularity of pistachio is mainly due to their nutritional components such as fatty acids and sterols since clinical studies, that evaluated the effect of a diet high in nuts on blood lipids, evidenced a decrease in the levels of low-density

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lipoprotein (Sabate et al., 1993; Soliman, 2012). In particular, pistachios have been reported to be the only nuts containing anthocyanins, the pigments responsible for the colour of pistachio kernel (Dreher, 2012) and the P.D.O. "Green pistachio of Bronte" is rich in anthocyanins, at a concentration greater than that contained in pistachios of several other geographical origins (Tomaino et al., 2010).

The health effect of pistachio is not attributable to a particular substance but to the simultaneous presence of various bioactive substance. In fact pistachio is a nutrient-dense nut with a heart-healthy fatty-acid profile as well as magnesium, potassium, copper, selenium, fiber, protein, folate, phytosterols, phenos, pigment, vitamin E, vitamin K and squalene (Dreher, 2012; Gentile et al., 2007; Giuffrida, Saitta, La Torre, Bombaci, & Dugo, 2006; Ryan, Galvin, O'Connor, Maguire, & O'Brien, 2006; Saitta, Giuffrida, La Torre, Potortì, & Dugo, 2009; Saitta, La Torre, Potortì, Di Bella, & Dugo, 2014).

Squalene (SQ) is an isoprenoid compound with 30 carbon atoms, containing six double bonds and it is present mainly in the oil of cod liver. For the first time it was isolated from the oil liver shark, but is widely distributed in nature in both the vegetable and animal tissues (Tsujimoto, 1916). In humans about 60% of SQ is absorbed from food and main source is olive oil which contains about 0.2–0.7% (Smith, 2000). It is transported through the serum in association with very low density lipoproteins and is distributed ubiquitously in human tissues, especially in the skin where it is a major component of lipid body surface area (Kelly, 1999). The average intake of SQ is 30 mg /day, however, when the consumption of olive oil is high or in combination with other foods that contain this molecule, the intake of SQ can reach 200–400 mg /day, as observed in Mediterranean countries (Saitta et al., 2009). SQ is essential for biosynthesis of steroids and triterpenes and in animal tissues it is an intermediate in endogenous cholesterol synthesis. For this reason, there is a concern that an increase of intermediate squalene, after intake of foods rich in this compound, can lead to cholesterol increase, which translates into a greater risk for development of atherosclerosis (Kohno et al., 1995).

On the contrary, it has been reported that SQ might be responsible of several beneficial effects reducing cholesterol and triglyceride levels in serum or protecting against a variety of cancers (Chan, Tomlinson, Lee, & Lee, 1996; Storm, Oh, Kimler, & Norton, 1993). This protective effect may be due to the possible antioxidant functions. The antioxidant and protective action is observed mainly in the skin in adults where SQ is one of the major components of skin surface lipids (Nicolaides, 1974). In fact, it has been demonstrated that SQ is not very susceptible to peroxidation and appears to function in the skin as a quencher of singlet oxygen, protecting human skin surface from lipidperoxidation due to exposure to UV and other sources of ionizing radiation (Kelly, 1999).

One of the first studies related to the SQ determination involved a colorimetric method (Rothblat, Martak, & Kritchevsky, 1962). Generally SQ in foods, oils and fats is determined by titrimetric (Association of Official Analytical Chemists (AOAC). Official method of analysis (OMA), 1999) or chromatographic procedures (Bondioli, Mariani, Lanzani, Fedeli, & Muller, 1993; Cert, Moreda, & Pérez-Camino, 2000; De Leonardis, Macciola, & De Felice, 1998) and some studies recommended SPE for sample preparation (Grigoriadou, Androulaki, Psmiadou, & Tsimidou, 2007; Popa, Băbeanu, Popa, Nită, & Dinu-Pârvu, 2015). The modern analytic methods used for SQ determination in various types of food are the gas-chromatography (GC) (Bueno, Casas, García, & González, 2005), alone or coupled with high performance liquid chromatography (HPLC) (Esche, Müller, & Engel, 2013; Grob, Artho, & Mariani, 1992; Villén, Blanch, Castillo, & Herraiz, 1998), or HPLC alone (Nenadis & Tsimidou, 2002; Sagratini et al., 2012) or coupled to mass spectrometry (HPLC/MS) (Di Stefano et al., 2012; Mountfort, Bronstein, Archer, & Jickells, 2007; Russo, Muzzalupo, Perri, & Sindona, 2010). In the existing HPLC or HPLC/MS methods, both normal and reversed phase were utilized (Manzi, Panfili, Esti, & Pizzoferrato, 1998; Mountfort et al., 2007; Ryan et al., 2006) and several authors proposed the use of UV, DAD and RI detector for SQ determination (Cortesi, Rovellini, & Fedeli, 1996; He, Cai, Sun, & Corke, 2002; Sun, Wiesenborn, Tostenson, Gillespie, & Rayas-Duarte, 1997). The direct SQ determination is complicated, and generally, the methods employed to extract SQ from foods involve a preliminary pre-treatment of the sample to eliminate the interfering substances. Commonly, pre-treatment consist of saponification and extraction of unsaponifiable, followed by a chromatographic separation by column with different fillers (Popa et al., 2015) or a preliminary fractional crystallization (Nenadis & Tsimidou, 2002). However, as before observed by Nenadis and Tsimidou (2002), most of the existing methodologies have been developed for the determination of other compounds and SQ was simply codetermined. In the last years, for the SQ isolation from seeds, the supercritical fluid extraction has been preferred, but this method is still expensive to industrial level (Popa et al., 2015). Now days, the official method for the determination of SQ involves sample saponification, extraction of the non-saponifiable matter with large volume of solvent, fractionation through chromatographic column and other treatment just before titration (AOAC. Official method of analysis, 1999). In the existing HPLC methods both official or not, the quantification of SQ by direct analysis is difficult and usually, pre-treatment involves relatively high volumes of organic solvent, high capital cost and long elution time.

The aim of our study was the development of a rapid, simple and efficient method for the determination of SQ in "green pistachio from Bronte" samples. In the present work, the SQ levels determination was performed in reversed phase and in the UPLC analytical method the mobile phase was optimized with respect to short time analysis and maximizing chromatographic resolution of the analyte. Furthermore, considering that the chemical composition of the pistachio seeds may vary depending on cultivar, rootstock and maturity at harvest, ecological conditions, growing areas, and horticultural practices (Kader, Heintz, Labavitch, & Rae, 1982; Seferoglu, Seferoglu, Tekintas, & Balta, 2006), the validated method was successfully applied to 29 "green pistachio from Bronte" samples to determine and compare SQ content trying to exploit this parameter as indicators of typicality.

2. Material and methods

2.1. Chemicals, reagents, and samples

Acetone and Acetonitrile were Optima UHPLC/MS and were Fisher Chemical products (Milan, Italy). Squalene (SQ) standard 98% and *n*-hexane and were purchased from Sigma-Aldrich (Milan, Italy). Stock solution of SQ was prepared in *n*-hexane at concentration of 10 mg mL^{-1} and stored at 18 °C; more dilute solutions were then prepared by appropriate dilution with *n*-hexane immediately before use. All the solutions were filtered through a non-sterile PTFE syringe filter 0.2 µm, purchased from Phenomenex (Bologna, Italy), before UPLC/PDA analysis. Cartridges Discovery DSC-Si Silica SPE (6 mL, 500 mg) were supplied from Supelco (Milan, Italy).

Twenty-nine fresh samples of pistachio (*Pistacia vera L.*, variety Bronte) were obtained from seven local growers belonging to *Consorzio del Pistachio di Bronte* (Catania, Italy). Pistachio nuts have been collected in early September in the year 2014. All samples were stored in the dark at 4 °C and analyzed within two months of collection.

2.2. Sample preparation

The nuts were shelled and pistachio seeds were accurately ground with a homogenizer. Then samples of 50 g were extracted with 200 mL *n*-hexane in a screw-cap flask, in the dark at room temperature (about 20 °C) for 30 min with the aid of ultrasound to facilitate homogenization of the nuts. The solvent was separated by settling and the residue was extracted again with 200 mL of fresh *n*-hexane. A total of three extractions were carried out (3 × 200 mL); further extractions did not allow recovery of appreciable quantities of oil. To the combined extracts were added 5 g of anhydrous Na_2SO_4 and then *n*-hexane was filtered. The solvent was removed under vacuum at room temperature; the oil obtained was flushed with a steam of dry nitrogen to volatilize the solvent residues. All the oil samples were stored at −5 °C before the clean-up.

2.3. Squalene clean-up

The lipophilic extracts were submitted to a solid-phase extraction and the optimized procedure was as follows. The oil sample extracted from pistachio seeds was weighted (~0.12 g), dissolved in 0.6 mL of *n*-hexane and loaded on the top of a 500 mg Supelco Discovery DSC-Si Silica column, previously washed with 5 mL of *n*-hexane. After that, the SPE column was connected to vacuum manifold and SQ was eluted with 10 mL of *n*-hexane at a flow rate of about 1 drop/s. The eluate was dried under vacuum at room temperature, redissolved in 1 mL of mobile phase, filtered through a 0.2 μm PTFE membrane filter and transferred into vial for immediate and subsequent UPLC/PDA analysis.

2.4. UPLC analysis

The SQ analysis was carried out using an *Acquity UPLC® Waters* liquid chromatography system equipped with a column heater, a photodiode array detector *ACQ-PDA*, a quaternary solvent manager *ACQ-QSM* and a sample manager *ACQ-FTN*, controlled by *Waters® Empower™* chromatographic software. In all analyses, an *Acquity UPLC® Waters BEH C18* column of 1.7 μm (2.1 × 50 mm), protected by 0.2 μm stainless steel *In-Line Filte*r with a *Holder Waters*, was used. Analyses were run at 40 °C, under isocratic condition, with a mobile phase composed of acetonitrile/acetone (60:40 v/v). The injection volume was 2 μl and the flow rate was 0.8 mL/min. SQ was detected and quantified using the PDA set at 217 nm wavelength.

3. Results and discussion

3.1. Analytical methods

In a preliminary phase of this study, all the parameters were subjected to optimization.

First, after initial trials to choose the appropriate solvent, *n*-hexane was selected as the extracting agent considering that *n*-hexane is an efficient solvent for extracting lipophilic substances and that squalene is a hydrocarbon with six non-conjugated double bonds. The influence of the extraction time and the solvent amount on the oil extraction behaviour was investigated, too. Isolation was than executed as described in the experimental part. The oils yields obtained were in the range 40.10–49.90 % (the mean of the twenty-nine samples was 44.98 ± 2.81 % of standard deviation).

Next, the stage of SPE purification was studied. Taking as the starting point the condition previously devised by other authors for the SQ determination in oil samples (Grigoriadou et al., 2007), the conditions were slightly modified to adapt the methods to the SPE column that we used and to the matrices that we studied. Different volumes of organic solvent were passed through to desorb SQ from SPE and the best result was achieved using 10 mL of *n*-hexane. Poor recoveries were obtained when smaller volumes were used and higher volumes diluted the sample only. The mixture extracted and purified, dried under vacuum at room temperature and redissolved in the mobile phase, was injected into the UPLC/PDA system.

In the UPLC analytical method, detection at 217 nm was considered more suitable and selective since at this wavelength, as illustrated in Fig. 1, the other compounds eluting near SQ are not detected. The mobile phase was optimized with respect to short time analysis and maximizing chromatographic resolution of the analyte. For this reason various elution phases and chromatographic conditions were studied. Finally, acetonitrile/acetone (60:40 v/v) were selected as this system gave better resolution, peak shape and stable baseline. The mobile phase velocity was also evaluated at 0.3, 0.5, 0.8 and 1 mL min^{-1} values and 0.8 mL min^{-1} was chosen as an optimal flow velocity. The selected isocratic chromatographic conditions allowed a very fast SQ determination; in fact this compound was well separated in ~0.54 min with good resolution. To test the kindness of the chromatographic separation, initially the mobile phase was injected three times and no peak was detected at the same SQ retention time. Then, for assessing the matrix effect and in order to establish the exact SQ retention time, three aliquots of a pistachio sample, whose SQ content had been previously determined, were spiked with different increasing amounts of SQ standard. The chromatographic analyses of these samples revealed a peak with gradually increasing area, but no interference was observed. Moreover, the peak purity was confirmed by comparing the PDA data of SQ standard with the peak of the respective analyte. In our chromatographic conditions were not found impurity or overlapping.

The last parameter examined was the column temperature. The values tested were 35 °C, 40 °C and 45 °C. At higher values the resolution of the chromatographic SQ peak did not improve and the column durability would decrease. The k' and the area versus temperature show that there were no differences among the temperature tested, although above 45 °C there was a slight tendency to decrease. A symmetrical peak of SQ standard was obtained at all temperatures and the peak area was slight higher at 40 °C. Thus, 40 °C was fixed for subsequent analysis.

The UPLC/PDA method was evaluated through validation parameters that included linearity, sensitivity, accuracy and repeatability according to a protocol set up initially in our laboratories and already adopted for the development of other analytical methods (Gentile et al., 2016; La Torre, Saitta, Potortì, Di Bella, & Dugo, 2010).

SQ quantification was done by measuring peak areas at SQ retention time and by comparing them with a calibration curve. A five-point calibration graph was obtained with 5, 10, 25, 50 and 100 mg L^{-1} standards. Calibration graph was achieved using linear regression of the least squares method and the peak response of each standard injection plotted against SQ concentration. Linearity was evaluated by the determination of the least square regression coefficients (r^2). The correlation coefficient was 0.9998. Calibration solutions were freshly prepared each day before the measurement. Each solution at different concentration was prepared in duplicate and injected in triplicate; the mean was obtained from all measurements.

Method sensitivity was evaluated by measuring the limits of detection (LOD) and of quantification (LOQ). The limits of detection (LOD) and of quantification (LOQ) were calculated using a signal-to noise ratio equal to 3.3 and 10, respectively (EURACHEM/CITAC guide, 2012) and were estimated with standards containing SQ at low concentration levels. LOD was 0.3 mg L^{-1} and LOQ was 1.0 mg L^{-1}.

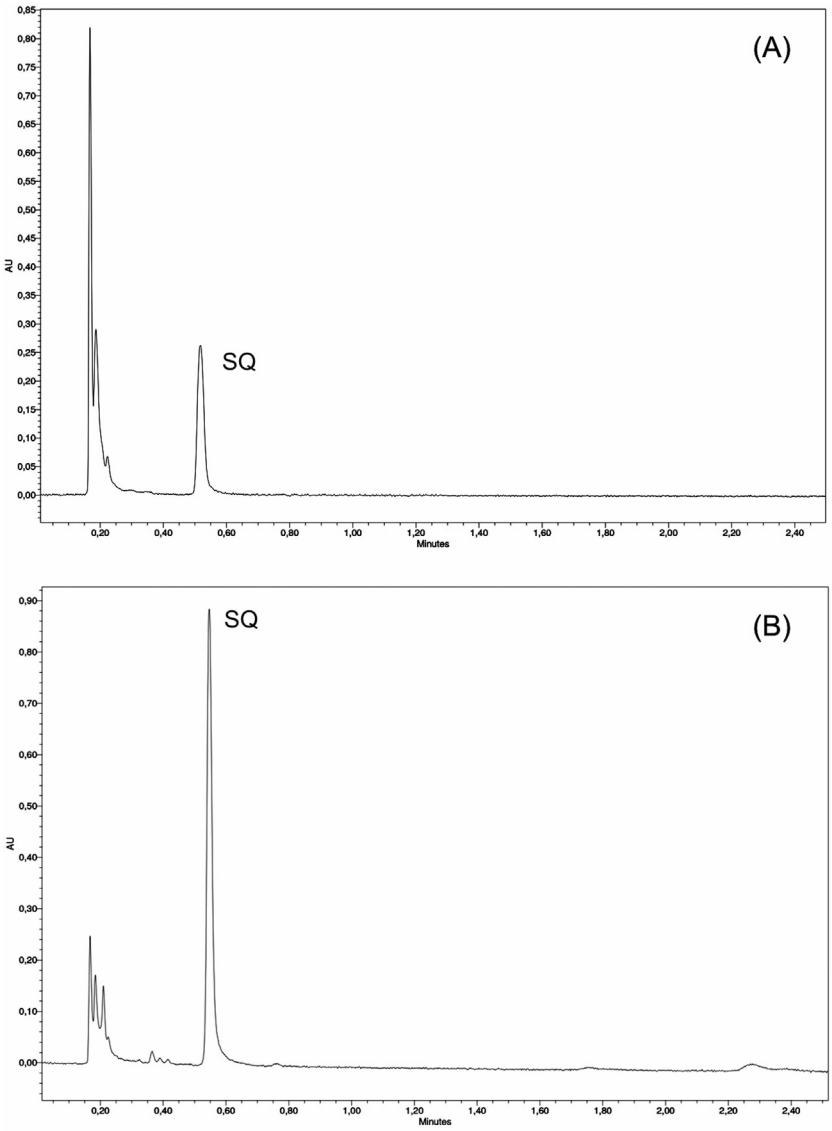

Fig. 1. UPLC-PDA chromatograms at 217 nm of (A) SQ standard solution and of (B) a "Green pistachio from Bronte" sample after SPE extraction.

The accuracy of the present method was evaluated by means of a spiking and recovery study on pistachio samples. Considering that "Green pistachio from Bronte" could contain significant levels of endogenous SQ by analogy to other nuts samples, the assessment of recoveries relied on the added amount with respect to the basal pistachio signals. Thus, the recovery of the full analytical procedure was carried out on 3 samples of ground pistachio nuts with a low SQ content, and was calculated by adding known amounts of SQ standard to pistachio samples whose SQ content had been previously determined. The recovery studies were carried out for two levels of standard SQ addition (25 mg L^{-1} and 50 mg L^{-1}, respectively) and five replication ($n = 5$). After addition, the samples were subjected to whole analytical procedure and the concentration of SQ in the samples was measured. As a result, peak area of SQ in the pistachio sample was subtracted from that corresponding to spiked pistachio as a measure of net SQ signal in the pistachio. The recovery (%) at both levels was satisfactory (92.8 ± 0.3 and 96.6 ± 0.1, respectively).

The precision of the method was expressed as the relative standard deviation (RSD) and two quality parameters (repeatability as intra-day and inter-day of retention time and peak area measurement) were determined. The intra-day repeatability of the method was assessed by performing five consecutive injections of SQ standards at two different levels of concentration addition (25 mg L^{-1} and 50 mg L^{1}, respectively) under the selected conditions and calculating the standard deviation. The same standards were also analyzed over a period of twelve successive days to determine the inter-day RSDs. The analytical precision assessed through the statistical results of the intra-day and inter-day determinations were 1.52 % and 3.37% for lower concentration and 1.00 % and 2.16% for high concentration, respectively. Retention time (RSD %) values for intra-day repeatability ($n = 5$) was lower than 0.2% and 0.98 % for inter-day repeatability ($n = 12$).

Therefore, the analytical characteristic can be considered satisfactory for the aim of the analysis.

3.2. Analysis of real samples of "Green Pistachio from Bronte"

After optimization of the analytical conditions, the method was applied for the determination of SQ in 29 "Green pistachio from Bronte" samples obtained from seven different local growers belonging to *Consorzio del Pistachio di Bronte* (Catania, Italy). Fig. 1 shows the chromatograms representative of SQ standard

Table 1
Occurrence of squalene in 29 "Green pistachio of Bronte" (P.D.O.) samples collected in Sicily from seven different local growers.

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7
	184,73 ± 1,87	226,34 ± 0,69	188,26 ± 1.87	207,25 ± 1.93	213,48 ± 3.09	195,50 ± 1.87	57,22 ± 0.69
	191,44 ± 0,75	188,69 ± 2,92	199,99 ± 1.01	210,43 ± 1.34	222,71 ± 2.48	192,46 ± 2.10	55,45 ± 0.75
	178,76 ± 1,92	195,30 ± 0.88	216,83 ± 1.03	194,84 ± 1.32	217,86 ± 2.08	192,50 ± 1.50	175,63 ± 1.87
	197,76 ± 2,42	178,75 ± 2.12	175,53 ± 1.57	206,83 ± 1.33	219,11 ± 2.40	196,64 ± 2.53	172,21 ± 1.57
					222,49 ± 2.10		
Mean	188,17	197,27	195,15	204,84	218,29	194,27	115,13

Values are expressed as mean values (mg $kg^{-1}$of oil) ± 95% confidence interval ($n = 3$).

and of a pistachio sample analyzed with the present UPLC-PDA acquisition mode.

SQ was identified in all the nuts analyzed and, as Table 1 shows, the quantitative results indicated that variation exist in SQ concentration from one sample to another. In general, it was found that SQ levels were significant, with the exception of two samples, the content of which is comparable with the few literature data (Esche et al., 2013; Wall, 2010). Mostly, the "Green pistachio from Bronte" showed a noticeable levels comparable with or higher than the SQ content of an unidentified macadamia cultivar (185 μg g^{-1} of oil) and hazelnuts (186 μg g^{-1} of oil) (Wall, 2010) SQ concentration ranged from 55.45–226.34 mg kg^{-1}. Except for samples from a farm (mean value 115.13 mg kg^{-1} of oil), the average SQ contents, in the examined pistachio samples, were quite comparable and ranged between 188.17 and 218.29 mg kg^{-1} of oil.

Current food databases contain little compositional data on SQ levels in pistachio nuts if compared to other compounds such as tocopherols, phenols and phytosterols which are attributed cardio-protective actions. The comparison with the few data highlighted that, with some exceptions, generally "green pistachio from Bronte" is characterized by a higher SQ content among variety examined so far.

4. Conclusion

The proposed method combines an efficient sample pretreatment and a rapid UPLC quantification of SQ in complex matrices such as pistachio. It is a quick and reliable analytical procedure and makes it possible to determine SQ without employing the saponification process. The sample pretreatment procedure, based on SPE with C18 cartridges has granted good extraction yields of SQ and a satisfactory sample purification. The use of cartridge for clean-up followed by UPLC has shown to be a technique with good analytical performance for squalene determination in pistachio nuts. Compared to other HPLC methods reported for the SQ analysis in food matrices (especially oil) good LOD was achieved (Nenadis & Tsimidou, 2002; Sagratini et al., 2012).

Of course, it is not possible to directly compare our method with similar ones, since the methods for SQ determination in natural source regarded mostly vegetable oils, and only few data focused pistachio and nuts. However, the proposed SPE-UPLC/PDA method is surely less expensive and more simple and rapid than the methods which use on line LC-GC (Esche et al., 2013).

The data provide new information on the composition of "Green pistachio from Bronte" and evidence that this variety contained significant amount of SQ. This is the first study in which SQ was extensively analyzed for "Green pistachio of Bronte". So, in order to improve the knowledge of the quality of this particular pistachio variety, to understand which are the most important markers that can be considered for an unambiguous geographical determination and for the safeguard of a foodstuff accredited by the European P.D.O. certificate, it would be of great help to process many other samples in different years of production. In this way, since the quality of pistachio nuts is usually related to its moisture content and biochemical composition (Crane, 1978) it would be possible to verify if the SQ content of "Green pistachio of Bronte" could be influenced by different climatic conditions.

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